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THE REVERSIBLE BINDING OF SOME AROMATIC AND CYCLIC COMPOUNDS TO BIOPOLYMERS *IN VITRO**

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ABSTRACT

A dialysis method is described for studying the weak, reversible interactions between a number of aromatic or polynuclear compounds, and non-diffusible biopolymers. As employed, the method is a semi-quantitative and rapid method for screening compounds for their potential binding properties.

It was found that melanin reversibly binds a large number of compounds, but that the binding may be selective amongst compounds of a similar chemical type. The physiologic implications of melanin-binding are not known. Trimethylpsoralen, a widely employed phototoxic agent, is found to bind primarily to melanin, DNA and lipoprotein. It is proposed that its phototoxic action may lie in its transfer of photon energy to lipid containing membranes with alteration of the latter.

The physical encounter between a small molecule and a biopolymer in solution may lead to: 1) a chemical reaction with the resulting formation of a chemical bond and an alteration in the identity of both reactants, 2) an elastic collision in which both species move apart unchanged, or 3) an interaction in which the two species are weakly attracted but without formation of a chemical bond. It is this latter situation with which we are concerned here.

We have reported earlier on the reversible binding of chlorpromazine (1) and iodoquine (2) to melanin pigment. The present work reports on the binding to other biopolymers as well as melanin and includes data on several compounds which have physiological or therapeutic roles.

MATERIALS AND METHODS

The technique employed consisted of obtaining the low molecular weight compounds radioisotopically labelled when possible. These were added to buffered solutions of biopolymers and allowed to stand over night at room temperature. These mixtures were then placed in dialysis bags, such that each contained 5 mg of the biopolymers in 10 ml of pH 6.85 KCl phosphate buffer. Immediately after placing the mixtures in the dialysis bags, a 0.5 ml aliquot was withdrawn, as the zero time sample. The dialysis bags were placed in separate one liter flasks, and dialysis was carried out at room temperature against a half liter of phosphate buffer on a laboratory shaker. To conserve buffer, this was changed daily instead of being continually in flow.

At varying time intervals thereafter, 0.5 ml aliquots were withdrawn from each of the dialysis bags, and the radioactivity of the aliquot was determined by gas flow-planchet counting for the ^{14}C labelled compounds and by scintillation counting for the tritiated compounds. In a few instances, when the tagged compounds could not be obtained, the concentration was determined spectrophotometrically.

The low molecular weight compounds were obtained in the following form: p-aminobenzoic acid carboxyl-^{14}C (Calbiochem. Corp.), chlormadinone-1α-^{3}H acetate, fluocinolone acetonide-^{14}C, 2α-methyldihydrotestosterone-1α-^{3}H, mestranol-9,11-^{3}H 3-methyl ether, paramethasone-7α-^{3}H acetate, estrone-9,11-^{3}H 3-methyl ether (Syntex Laboratories), chlorpromazine-^{35}S (Radiochemical Centre, Amersham), tetracycline-7-^{3}H, iodoquine-^{131}I (New England Nuclear Corp.), trimethylpsoralen-^{3}H (through the courtesy of Dr. Kurt Kaufmann, Kalamazoo College), 2-naphthoic acid carboxyl-^{14}C, 3-indolyl acetic acid, 1-^{14}C (Nuclear Chicago), chlortetracycline, demethylchlortetracycline (Lederle).

The biopolymers employed were: DNA, calf thymus (Worthington Biochemicals), RNA (yeast) highly polymerized (Mann Research Laboratories), egg albumin, 2x crystallized (Calbiochem.), beta lipoprotein (human) Fraction III-D (Nutritional Biochemicals), and the melanin used was prepared from squid ink by the cold HCl method of Nicolaus (3). The biopolymers were made up

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in 10 ml of buffer (0.1 N KCl with 0.05 N phosphate buffer, pH 6.85). One dialysis bag containing only the buffer and the low molecular weight compound, served as control.

RESULTS

The radioactivity remaining in each dialysis bag was plotted as a function of dialysis time on a semilog plot, since ideal diffusion should give an exponential rate of loss of radioactivity from the dialysis bag. The results are shown in Figures 1 to 10. A number of the runs were repeated, and a satisfactory reproducibility was demonstrated.

The interpretation of these dialysis curves seems relatively straightforward, and is given in the discussion below. As a rough measure of the interaction between the various small molecules and the biopolymers, we may take the ratio of the radioactivity remaining in a particular dialysis bag to that remaining in the control dialysis bag after an arbitrary interval of time. The results for a number of pairs of interactants are given in Table I.

DISCUSSION

Because of our primary interest in melanin binding compounds (1, 2) this semi-quantitative dialysis method was developed as a screening procedure for detecting such behavior.

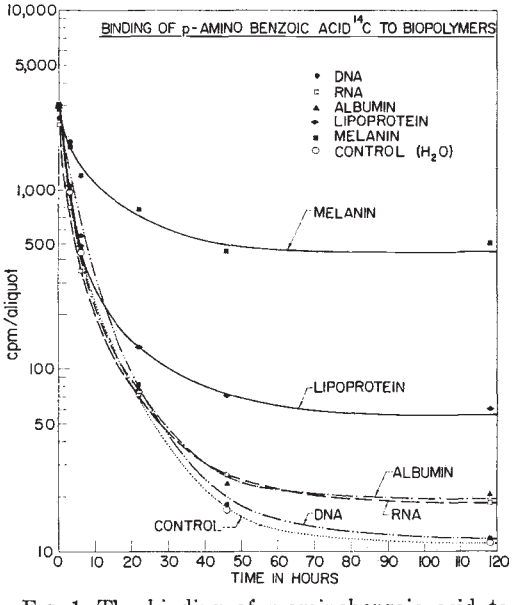

FIG. 1. The binding of p-aminobenzoic acid to several biopolymers (4.6 μmoles/mg of polymer).

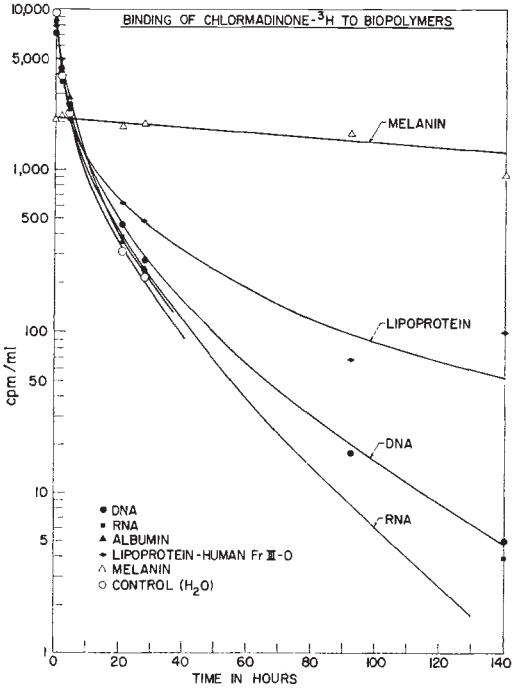

FIG. 2. The binding of chlormadinone to several biopolymers (1.66×10^{-3} μmoles/mg of polymer).

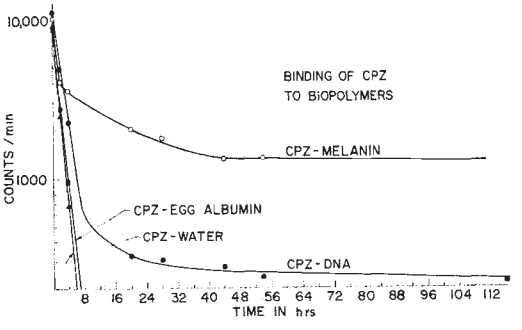

FIG. 3. The binding of chlorpromazine to several biopolymers (96 μgm/mg of polymer).

Squid melanin was employed because it can be obtained in relatively pure form using somewhat milder procedures than are necessary with mammalian melanins. Squid and mammalian melanins have been shown by Nicolaus, using chemical methods, to be of the indole type (4), and by physical studies (5, 6) to be indistinguishable. The employment of biopolymers other than melanin was undertaken because of their intrinsic interest and their potentialities for drug binding.

Two experimental features may be com-

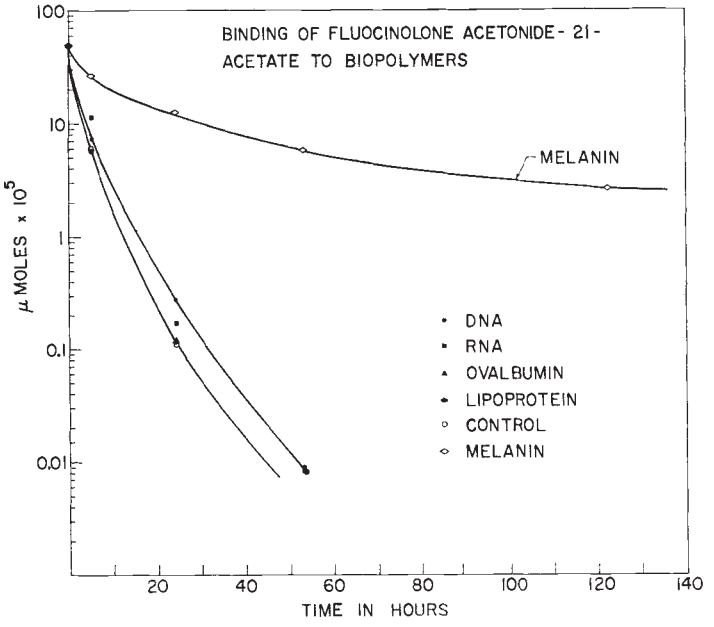

FIG. 4. The binding of fluocinolone acetonide-21-acetate to several biopolymers (0.02 μmoles/mg of polymer).

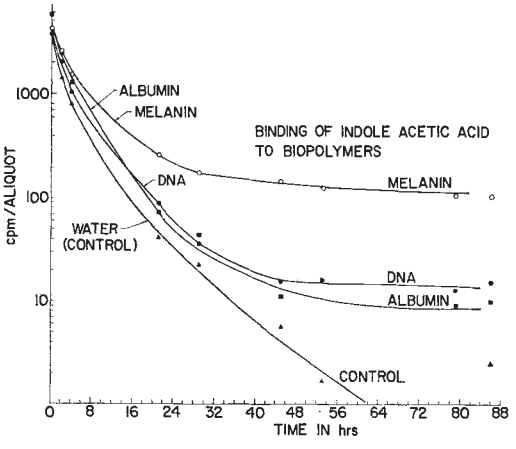

FIG. 5. The binding of indole acetic acid to several biopolymers (0.55 μgm/mg of polymer).

mented upon which apply to all of the individual experiments. In this dialysis method, two extreme types of behavior are predictable: if the compounds chemically bind to the biopolymers, a stable, nondiffusible product would result and there would be no further loss of radioactivity from the dialysis bag once the unbound fraction was dialyzed out. If there were no interaction between the two, the tagged compound would diffuse out at approximately the same rate as the control. If the tagged compound diffuses out at a significantly slower rate than in the control, it would be concluded that the compound is interacting with the polymer.

One feature that is immediately obvious from the diffusion curves is the general non-ideality of the controls. In the representation used, the tagged compounds would be expected to diffuse out at an exponential rate in the controls, and the curves should be straight lines. While this is true initially, it later becomes apparent in many of the runs that the rate of loss of activity is decreasing. There are several possibilities that could account for this behavior: 1) non-diffusibility of a portion of the tagged compound used due to particulate matter, microbial contamination of the system, or polymerization or aggregation of the tagged compound; 2) insoluble tagged impurities in the original sample; or 3) binding to the dialysis bag itself. The first group of possibilities were eliminated or reduced by carrying out all dialysis under sterile conditions, and by using low environmental light levels to reduce photopolymerization. In order to determine whether the original tagged materials contained non-diffusible label, several runs were made in which the tagged compounds were

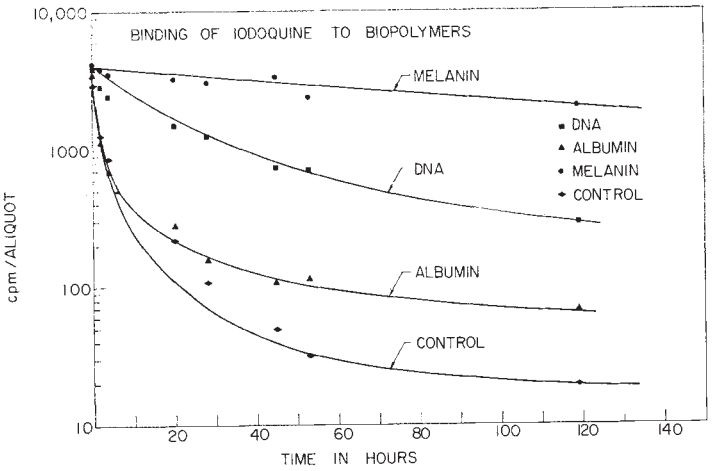

FIG. 6. The binding of iodoquine to several biopolymers (0.66 μgm/mg of polymer).

first dialyzed with the same dialysis tubing. The labeled compound in the dialysate was then immediately redialyzed and the rate of loss of radioactivity from the dialysis bag was determined. The same non-exponential behavior was found. It is our conclusion that a fraction of the labeled compound may be reversibly bound to the cellulosic material of the dialysis bag, so that when desorption subsequently occurs and the label is released into the inside solution, this acts as a "virtual" source of label and accounts for the observed results.

In addition, the melanin experiments cannot be compared with those of the other polymers without a further comment. This is because the latter form true solutions under our experimental conditions, while the melanin particles do not. Some of the melanin slowly settles in the dialysis bags, so that a portion of the tagged compound may be physically trapped between melanin particles at the bottom of the bag and impeded in its diffusion outward. On the other hand, the tagged compound in the supernatant would then be less impeded in its outward diffusion because of the lowered melanin concentration there, and this effect should tend to offset the other. A number of techniques for keeping the melanin in suspension were tried, (e.g., bubbling a N_2 stream through the suspension) and the results obtained in these experiments were not significantly different from the data given here. It was concluded that the two offsetting factors largely cancelled each other.

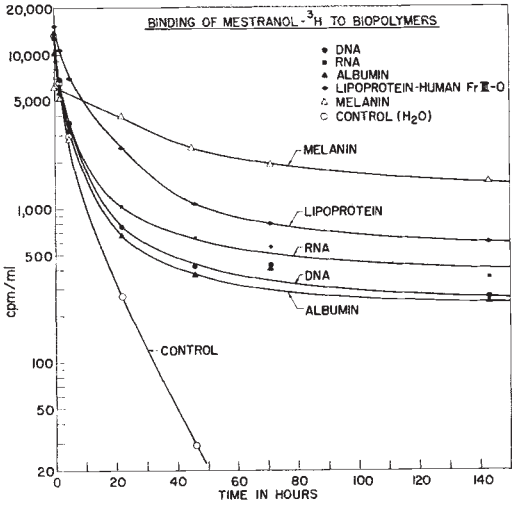

FIG. 7. The binding of mestranol to several biopolymers (3×10^{-3} μmoles/mg of polymer).

A third question concerns whether the biopolymers have a fixed number of binding sites for the small molecules or whether the adsorption of the latter are governed only by the laws of thermodynamics. If the former were true, the amount of radioactivity remaining after prolonged intervals would be independent of the amount originally added. In order to evaluate this, separate experiments were run empoloying different concentrations of the small molecule. Figure 11 shows the results with 2 α methyldihydrotesterone and melanin in which the steroid concentration was varied over two orders of magnitude and

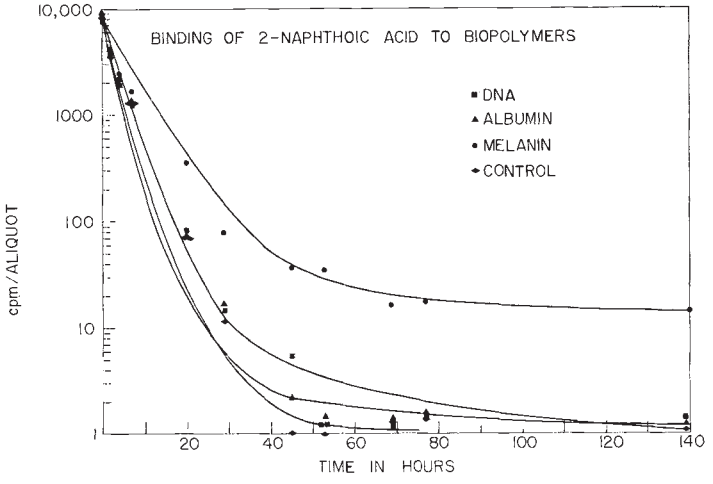

FIG. 8. The binding of 2-naphthoic acid to several biopolymers (3.7 μgm/mg of polymer).

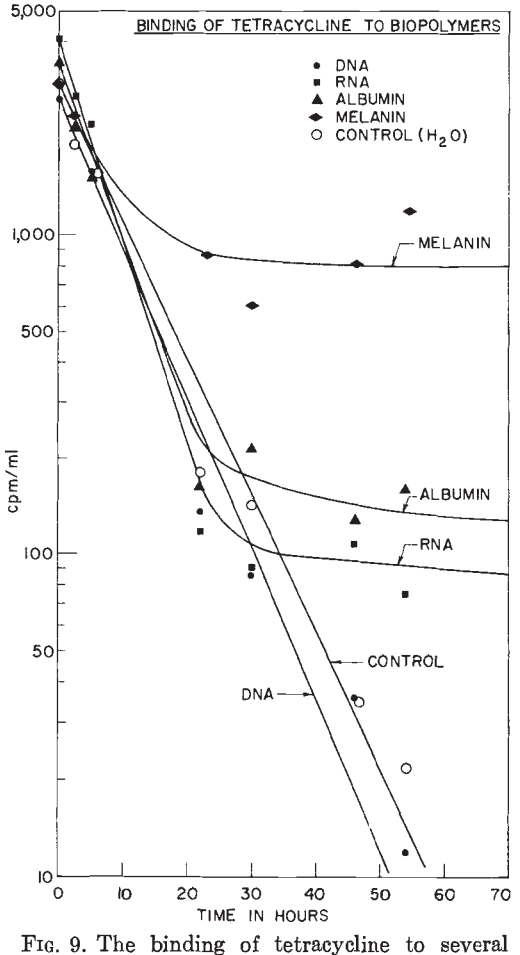

FIG. 9. The binding of tetracycline to several biopolymers (6.3×10^{-4} μmoles/mg of polymer).

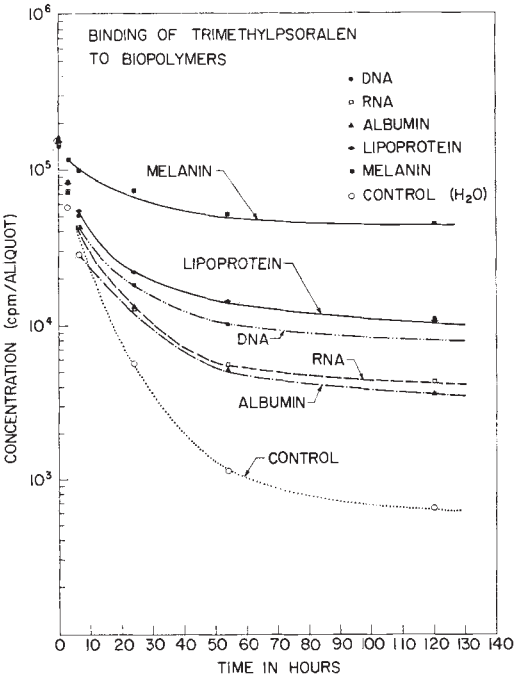

FIG. 10. The binding of trimethylpsoralen to several biopolymers (0.03 μgm/mg of polymer).

the amount of melanin kept constant. It is immediately apparent that the amount of radioactivity remaining is proportional to the amount of the steroid originally added. For this pair of interactants the bound fraction is thus proportional to the amount present and

the binding energy as indicated by the slopes, are independent of concentration.

Despite the difficulties of comparing melanin directly with the other polymers, it clearly intereacts strongly with a number of compounds in addition to the cases of chlorpromazine, chloroquine and iodoquine, which have been reported earlier in either *in vitro* or *in vivo* studies. Since it interacted so strongly with a variety of molecules, it suggested the behavior of a non-specific adsorbant such as activated charcoal. It was interesting, therefore, to find that some compounds, such as certain steroids, (paramethasone, estrone) were bound very weakly, if at all. On the other hand, other steroids (fluocinolone acetonide acetate, mestranol, etc.) were strongly bound.

TABLE I

Approximate binding ratios, i.e., ratio of radioactivity remaining in the dialysis bag to that remaining in the control after 50 hours of dialysis

	DNA	RNA	Albumin	Lipoprotein	Melanin
p-Aminobenzoic acid	1	1.5	1.5	4.3	30
Chlormadinone	~1	~1	~1	4	30–100
Chlorpromazine	5	—	~1	~1	25–50
Fluocinolone acetonide acetate	2	2	~1	~1	1000
Fluocinolone acetonide	~1	~1	2	2	5–20
Indole acetic acid	5	—	5	—	50
Iodoquine	20	—	3	—	85
Mestranol	20	30	20	50	110
Naphthoic acid	4	—	2	—	35
Paramethasone acetate	1	2	1	1	100
Tetracycline	~1	4	7	—	40
Trimethylpsoralen	9	5	5	10	35

Because the melanin sample used had been chemically purified, the physiochemical surface properties of the pigment particles may not closely resemble those of the melanosome *in vivo*, and biological implications of these results should not be drawn from these data alone. It is noteworthy, however, that for those compounds which have been studied both by the dialysis method and *in vivo* (chlorpromazine and iodoquine), the melanin binding behavior has been similar (1, 2).

Although it has been proposed (7) that melanin may have a physiologic photoprotective role by acting as a trap for light-induced free radicals, there is at present little direct evidence to support this opinion. On the other hand, the results of Table I indicate that melanin strongly binds a variety of compounds.

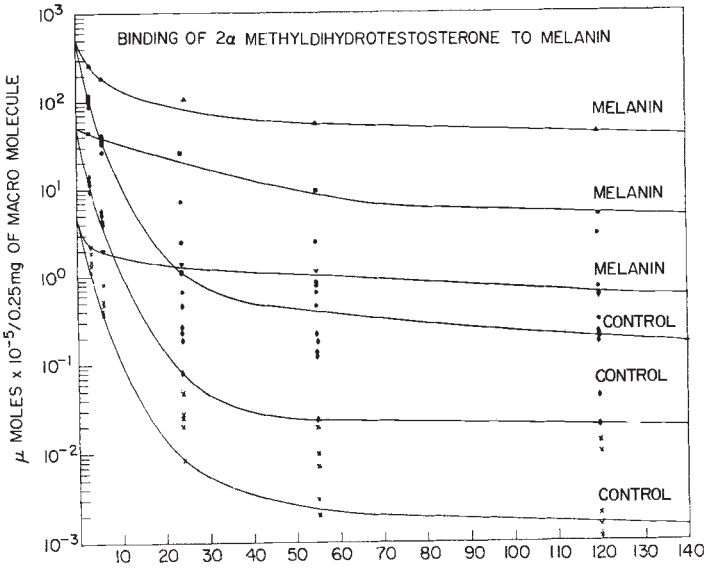

FIG. 11. The binding of 2α-methyldihydrotestosterone to melanin at different concentrations.

Whether this implies a physiological role remains to be established.

The interactions of chlorpromazine and especially chloroquine with nucleic acids have been widely studied (8, 9) and need no comment here. One interesting finding, however, is the interaction shown by trimethylpsoralen, particularly with respect to DNA and lipoprotein. The mechanism of action of this photosensitizer is at present speculative, but it may be assumed that a prerequisite step is its binding to a critical molecule or structure (perhaps the membrane of a lysosome or capillary). When an incident photon, with an energy corresponding to the trimethylpsoralen absorption band, is then absorbed by this molecule, the energy may be transferred by a non-radiative process to this critical target thereby altering it. Two such potential targets are suggested by the binding data presented here: DNA and lipoprotein. Because of the immediacy of the phototoxic response, it may be proposed that tripsoralen functions pharmacologically by the photosensitization of membranes. Its photoreactivity with DNA, which has been studied by Musajo and coworkers (10, 11), might seem more likely to be associated with delayed and chronic effects, such as light-induced carcinogenesis.

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